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14P1

The electron transfer pathway of Type-C oxygen reductase from *V. cholera*

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There are 3 major families of heme-copper respiratory oxygen reductases, A-, B- and C-type enzymes. The eukaryotic mitochondrial cytochrome aa_3 is a Type-A enzyme. A number of human pathogens contain a Type-C oxidase, also known as cytochrome cbb_3 [1]. Protons, electrons and O_2 must each have access to the enzyme active site, where O_2 is reduced to water. We recently found the natural electron donor for cbb_3 oxidase of *Vibrio cholerae*. The diheme cytochrome c_4 can donate electrons to cytochrome cbb_3, supporting steady state oxidase activity of at least $300e^{-1}$ [2]. However the sequence of electron transfers within the redox-active hemes of cytochrome cbb_3 is not clear. Looking at the X-ray crystal structure of the cbb_3 oxidase, subunit III (CcoP) appears to be a candidate for the initial acceptor of the electron from cytochrome c_4 [3]. Subunit III contains two N-terminal transmembrane helices and a periplasmic globular domain containing two cytochrome *c* moieties. Subunit II (CcoO) contains one cytochrome *c*. Previous studies in our laboratory used point mutations to perturb each of the three cytochrome *c* components of the enzyme, and showed that each cytochrome *c* is essential for function. In this work, we examine the electron transfer pathway after performing two different procedures: 1) removing subunit III entirely, and 2) truncating just the periplasmic, hydrophilic domain. Deletion of subunit III resulted in no enzyme being found in the membrane. Deletion of just the periplasmic domain resulted in a stable complex containing subunits I and II, but oxidase activity is significantly reduced. This data shows that in *V. cholera*, the periplasmic domain of subunit III is required for oxidase activity as the likely entry point for electrons from cytochrome c_4 and that the transmembrane helices appear to be required for stability and/or assembly. This situation is similar to that found in the A-type cytochrome *c* oxidases in which the hydrophilic domain of subunit II is the docking site for cytochrome *c* and a critical glutamic residue in subunit II is the entrance to the K-channel for protons.

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14P2

Mutational analyses of D-pathway of bovine heart cytochrome *c* oxidase suggest that the pathway does not transfer the pumping protons

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X-ray and mutagenesis analyses of bovine heart cytochrome *c* oxidase strongly suggest that protons are pumped through H-pathway [1–3], where Asp51 essential to pumping is not conserved in bacteria, lower eukaryotes and plants. On the other hand, it has been proposed that D-pathway conveys both water-forming and pumping protons based on the following mutation results of bacterial D-pathway; Asn98Asp and Asn163Asp mutations (bovine numbering) abolish proton pumping with keeping high O_2 reduction activity, while Asp91Asn and Glu242Gln mutations (bovine numbering) abolish O_2 reduction activity. Bacterial and bovine D-pathway structures closely resemble to each other and amino acid residues essential to bacterial D-pathway are conserved in bovine in contrast to H-pathway. Structural similarity between two D-pathways implies proton pumping through bovine D-pathway. Here, we report mutagenesis analysis of bovine D-pathway employing HeLa cell bovine/human hybrid enzyme expression system we have developed [1,2]. Asn98Asp and Asn163Asp mutations showed no influence on both proton pumping and electron transfer activities, contradicting to the bacterial results. Sequencing of 13-subunit genes or cDNAs of the hybrid enzymes revealed no other mutations, which excludes any possibility for back mutation to restore the enzymatic activity. Asp91Asn and Glu242Gln mutations abolished completely the enzymatic activity. These results support that bovine D-pathway is for water-forming protons, not for pumping protons.

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14P3

Direct evidence for *Acanthamoeba castellanii* alternative oxidase gene function

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Alternative oxidase (AOX) is a cyanide-resistant ubiquinol terminal oxidase present in mitochondria of many phyla like plants, fungi and *protists*. A small non-photosynthesizing free-living amoeba *Acanthamoeba castellanii* contains a plant-type mitochondrial respiratory chain with additional electron carriers, including AOX (AcAOX) that consumes mitochondrial reducing power without energy

conservation. It has been shown that AcAOX may play a role in the energetic status of the amoeba cell (by decreasing the yield of ATP synthesis) and in attenuating reactive oxygen species production. AcAOX is stimulated by purine nucleotides, except ATP that has an inhibitory effect on AcAOX activity.

A gene for AcAOX has been identified but there has been no direct functional evidence that it encodes cyanide-resistant mitochondrial oxidase. Using *AcAOX* cDNA sequence we performed relative quantification real time PCR and found that changes in *AcAOX* mRNA levels during growth of *A. castellanii* batch culture follows the pattern of changes in AcAOX protein levels and the enzyme activity.

In order to confirm an enzymatic function of the AcAOX gene product, we performed oxygen consumption measurements of *Escherichia coli* DH5α strain transformed with pDrive containing the *AcAOX* gene. In transformed bacterial cells, the cyanide-resistant benzohydroxamate-sensitive respiration was observed, indicating function of active AcAOX enzyme. Moreover, the activity was stimulated by GMP and inhibited by ATP, as it has been previously shown for AcAOX in isolated *A. castellanii* mitochondria. The AcAOX protein was also identified in transformed bacterial cells by immunodetection.

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14P4

Cytochrome *bd*-II from aerobic respiratory chain of *Escherichia coli* is a proton-motive force generator

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Escherichia coli is known to couple aerobic respiratory catabolism to ATP synthesis by virtue of the three primary proton-motive force (*pmf*) generators: NADH dehydrogenase I (NDH-I), cytochrome bo_3, and cytochrome *bd*-I. The non-pumping, copper-lacking three-heme *bd*-type oxidase couples oxidation of quinol to reduction of O_2 to H_2O [1,2]. Nevertheless, *E. coli* mutant strains deficient in NDH-I, bo_3, and *bd*-I can grow under aerobic and glucose-limited conditions, although its sole terminal oxidase cytochrome *bd*-II was proposed to be non-electrogenic. In the present work, the ability of cytochrome *bd*-II to generate *pmf* is reexamined. Absorption and fluorescence spectroscopy and oxygen pulse methods all show that in the steady-state, cytochrome *bd*-II is capable of producing both electrical and chemical components of *pmf* yielding H^+/e^- ratio of 0.94 ± 0.18; this is sufficient to drive ATP synthesis and transport of nutrients. Microsecond time-resolved, single-turnover electrometry provides evidence for a molecular mechanism of the proton motive force production [3]. The ability to induce cytochrome *bd*-II biosynthesis allows *E. coli* to remain energetically competent under a variety of environmental conditions, particularly at the extremely low oxygen pressure and carbon and phosphate starvation. Work supported by RFBR (grant 11-04-00031-a).

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14P5

Proton pumping in cytochrome *c* oxidase — An explicit gating mechanism based on experimental information, electrostatic considerations and QM calculations

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In cytochrome *c* oxidase, the terminal enzyme in the respiratory chain, protons are pumped against the electrochemical gradient over the mitochondrial membrane, to store the energy released when molecular oxygen is reduced to water. A gating mechanism for the protons, involving a positively charged transition state was suggested based on electrostatic interpretations of kinetic experiments [1–4]. The predictions from that analysis have been tested using hybrid DFT with large chemical models (about 400 atoms). The proposal that a positively charged transition state for proton transfer is needed to obtain a gate is confirmed by the QM calculations. It is shown that a few critical relative energy values from the earlier studies are reproduced with quite high accuracy using this large model. Examples are the low forward barrier for proton transfer from the N-side of the membrane to the pump-loading site when the electron transfer cofactor heme *a* is reduced, and the corresponding high back leakage barrier when heme *a* is oxidized. At present MD simulations on even larger models are performed to give new starting structures for the QM calculations and thereby further validate the calculated energetics.

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14P6

Cytochrome *bd* oxidase: Direct observation of the catalytic intermediates at steady-state

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